nature medicine

Inhibition of respiratory syncytial virus infection with intranasal siRNA nanoparticles targeting the viral NS1 gene

Weidong Zhang[1,2], Hong Yang[2], Xiaoyuan Kong[1,2], Subhra Mohapatra[3], Homero San Juan-Vergara[2], Gary Hellermann[1,2], Sumita Behera[4], Rajeswari Singam[4], Richard F Lockey[1,2] & Shyam S Mohapatra[1,2]

Respiratory syncytial virus (RSV) infection is one of the major causes of respiratory tract infection for which no vaccine or antiviral treatment is available. The RSV NS1 protein seems to antagonize the host interferon (IFN) response; however, its mechanism is unknown. Here, we used a plasmid-borne small interfering RNA targeting the NS1 gene (siNS1) to examine the role of NS1 in modulating RSV infection. RSV replication was reduced in A549 cells, but not IFN–deficient Vero cells, transfected with siNS1. siNS1 induced upregulated expression of IFN-β and IFN-inducible genes in A549 cells. siNS1-transfected human dendritic cells, upon RSV infection, produced elevated type-1 IFN and induced differentiation of naive CD4+ T cells to T helper type 1 (TH1) cells. Mice treated intranasally with siNS1 nanoparticles before or after infection with RSV showed substantially decreased virus titers in the lung and decreased inflammation and airway reactivity compared to controls. Thus, siNS1 nanoparticles may provide an effective inhibition of RSV infection in humans.

RSV is a major viral respiratory pathogen and produces an annual epidemic of respiratory illness causing bronchiolitis and otitis media in infants and young children[1,2] and pneumonia in adults and the elderly[3,4]. During 1991–1998, RSV was associated annually with over 17,000 deaths[5]. Immunodeficiency, cardiac arrhythmia and congenital heart disease are risk factors for more severe diseases with RSV infection[6–8]. An effective vaccine is not available for RSV and may not even be practical given the immunocompromised state of the target high-risk population, the incomplete immunity developed even by natural RSV infection and its short incubation period[9,10]. Consequently, DNA-based prophylactics are under investigation.

RSV is the prototypic member of the genus *Pneumovirus* and is an enveloped, nonsegmented, negative-stranded RNA virus. The RSV genome of approximately 15,200 nucleotides is transcribed into 10 transcripts encoding 11 distinct proteins, including 2 nonstructural proteins, NS1 and NS2, which are expressed from separate mRNAs encoded by the first and second genes, respectively[11]. Deletion of either NS1 or NS2 severely attenuates RSV infection *in vivo* and *in vitro*, indicating that NS proteins have an important role in viral replication[12–15]. Furthermore, repeated RSV infections are common as a result of the incomplete immunity caused by natural infection, the basis of which is poorly understood[16]. RSV infection was shown to be associated with a predominantly T helper type 2 (T_H2)-like response in infants[17], although results of studies in children have been inconsistent. Hence, RSV is considered a predisposing factor for the development of allergic diseases and asthma[18,19].

IFNs attenuate RSV replication and also have therapeutic value against allergic diseases, including asthma[20–22]. We and others have developed *in vivo* intranasal gene delivery approaches using nanoparticles composed of chitosan, a natural, biocompatible and biodegradable polymer[21–24]. Because bovine and human RSV NS1 seem to antagonize the type-1 IFN-mediated antiviral response[25–28], we reasoned that blocking NS gene expression might attenuate RSV replication and provide an effective antiviral and immune enhancement therapy. The short interfering RNA (siRNA) approach has proven effective in silencing a number of genes of different viruses[29]. Here we used this approach to examine the potential and mechanism of siNS1 to inhibit RSV replication in cultured human epithelial cells, modulate immunity against RSV in human dendritic cells and attenuate RSV infection in mice. The results show that siNS1-mediated silencing of the NS1 gene substantially suppresses RSV replication and modulates host immunity to RSV infection compared with control groups.

RESULTS

siNS1 inhibition of recombiant RSV infection

Two different siRNA oligonucleotide sequences for RSV NS1, siNS1 and siNS1a, and control siRNAs against HPV18E7 (siE7) and influenza virus PB2 (siPB2) were designed and cloned into the pSMWZ-1 vector[30]. Analysis of EGFP expression in A549 cells cotransfected with pEGFP and siNS1, siNS1a, siE7 or siPB2 showed that none of the siRNAs silence the EGFP gene (data not shown). Immunoblotting results showed that pretransfection of A549 cells with siNS1, but not siE7, substantially reduced the expression of NS1 proteins (**Fig. 1a**) but not that of other viral proteins (data not shown) at 24 h after infection with a recombinant RSV expressing GFP (rgRSV)[31]. To test whether siNS1 attenuates

[1]Division of Allergy and Immunology–Joy McCann Culverhouse Airway Disease Research Center, Department of Internal Medicine, University of South Florida, MDC-19-Rm 2536, 12901 Bruce B. Downs Boulevard, Tampa, Florida 33612, USA. [2]James A. Haley Veterans Hospital, 13000 Bruce B. Downs Boulevard, Tampa, Florida 33612, USA. [3]Department of Interdisciplinary Oncology, H. Lee Moffitt Cancer Center, 12902 Magnolia Drive, Tampa, Florida 33612, USA. [4]TransGenex Nanobiotech Inc., UTC-II, 3650 Spectrum Boulevard, Suite 170, Tampa, Florida 33612, USA. Correspondence should be addressed to S.S.M. (smohapat@hsc.usf.edu).

Published online 26 December 2004; doi:10.1038/nm1174

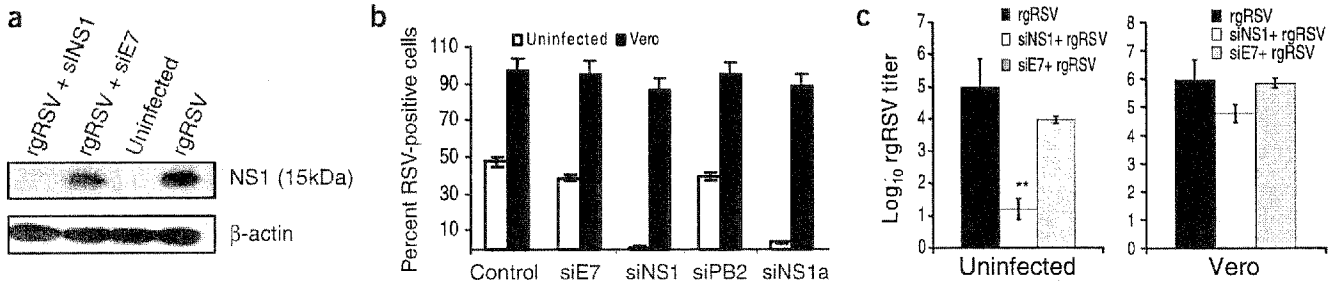

Figure 1 siNS1 inhibits rgRSV infection. (**a**) Immunoblot analysis of NS1 protein expression at 24 h after infection with rgRSV. (**b**) Flow cytometry analysis of rgRSV-positive uninfected cells and Vero cells, respectively. Control versus siNS1 and control versus siNS1a, $P < 0.01$. (**c**) Measurement of virus titer using plaque assay. Data are the averages of two independent experiments; $^{**}P < 0.01$ when compared with control group.

virus infection, we transfected A549 cells and type-1 IFN–deficient[32] Vero cells with the siNS1, siNS1a or control siRNAs, and then infected them with rgRSV. The results of flow cytometry showed a significant ($P < 0.01$) decrease in the percentage of cells expressing EGFP. In marked contrast to A549 cells, siNS1 and siNS1a did not decrease viral replication in Vero cells as compared to controls (**Fig. 1b**). Furthermore, plaque assays for RSV titers in culture supernatants indicated that siNS1 significantly decreases ($P < 0.01$) rgRSV titer compared to controls in A549 but not Vero cells (**Fig. 1c**). Plaque assays using siNS1a gave results similar to those from siNS1 (data not shown). Together, these results indicate that siNS1 attenuates RSV infection in a gene-specific fashion, and this attenuation may involve modulation of the type-1 IFN pathway by NS1.

Mechanism of siNS1-mediated upregulation of the type-1 IFN pathway

The finding that RSV infection of A549, but not Vero, cells is affected by siNS treatment suggests a role of NS1 protein in the promotion of RSV infection by inhibiting the type-1 IFN pathway. To verify whether NS1 decreases type-1 IFN, we examined the expression of IFN-β by immunoblotting. The results show that A549 cells transfected with siNS1 or siNS1a, upon RSV infection, produce substantially increased amounts of IFN-β, compared to the different controls, including unrelated siRNA with no homology to mammalian genes (siUR) (**Fig. 2 a,b**). To further examine the role of NS1 in regulating the IFN pathway, we isolated RNAs from control and siNS1-transduced cells and subjected them to microarray analyses. The results show that siNS1 treatment increased the expression (≥6-fold change) of 25 IFN-inducible genes compared to rgRSV infection alone (**Table 1**), and we investigated the expression of a number of altered genes by western blotting. The results show that the phosphorylated STAT1 (Ser727), STAT1, IRF1, IRF3, ISGF-3γ and MxA proteins were upregulated after siNS1 inhibition (**Fig. 2c**). To determine whether NS1 affects STAT1 and IRF1 translocation in A549 cells, we infected transfected cells with wild-type RSV (MOI = 0.1), fixed them 3 h later, permeabilized them and stained them with appropriate antibody. Cells treated with siNS1 showed significantly higher nuclear localization of phosphorylated STAT1 and IRF1 compared to controls ($P < 0.05$ and $P < 0.01$, respectively; **Fig. 2d,e**), suggesting that the NS1 protein blocks trafficking of these proteins into the nucleus.

Silencing NS1 polarizes human DCs toward a T_H1-promoting phenotype

Monocytes isolated from human peripheral blood were cultured with requisite cytokines to test whether siNS1 expression affects RSV-infected dendritic cell (DC) activity. Thus, we measured the concentration of IFN-α and IFN-β in the supernatants from cultured, infected, monocyte-derived DCs transfected with siNS1 or control siRNA. The data show that siNS1 treatment induced a significantly higher ($P < 0.05$) production of both type-1 IFNs in infected DCs than the control siRNA (**Fig. 3a**). Furthermore, to assess the effect of siNS1-treated DCs on T-cell function, we cultured allogenic naive $CD4^+$

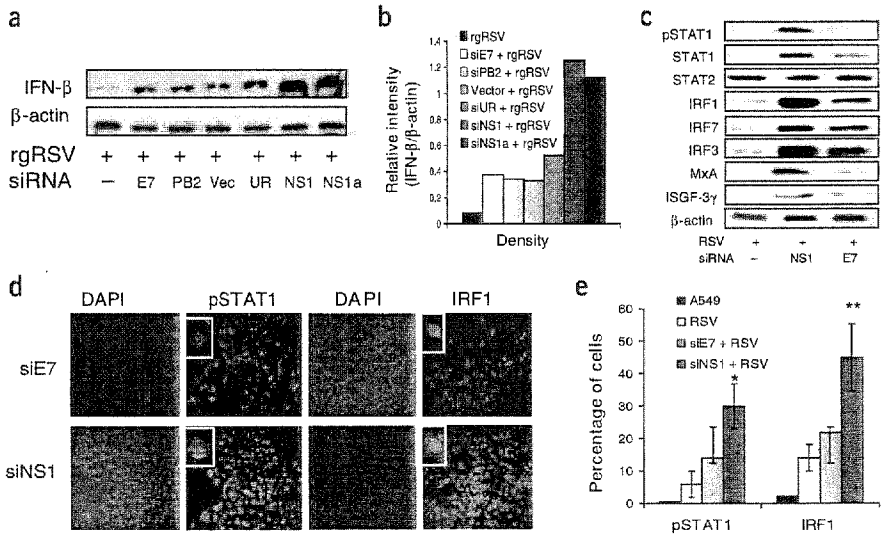

Figure 2 siNS1-mediated attenuation of RSV infection involves upregulated expression of IFN-β and IFN-inducible genes in infected A549 cells. (**a**) Immunoblot analysis of IFN-β protein expression at 24 h after infection with rgRSV. (**b**) Protein bands were scanned using the Scion image system (US National Institutes of Health) to quantify data in **a**. (**c**) Immunoblot analysis of the expression of IFN-inducible genes 3 h after RSV-infected A549 cells. For each, the results of one experiment of two performed with similar results are shown. (**d**) NS1 protein prevents nuclear import of IRF1 and STAT1. The nuclear localization of the IRF1 and STAT1 proteins in A549 cells was examined by indirect immunofluorescence using corresponding antibody and visualized and photographed under a fluorescent microscope. (**e**) The percentage of positive cells was determined from 100 cells per field. Data are mean ± s.d. from two separate experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to control.

T cells with RSV-infected DCs treated with or without siNS1. The results of intracellular cytokine staining showed an increase in IFN-γ and a decrease in IL-4 secretion in naive $CD4^+$ T cells after they were cultured with siNS1-treated, RSV-infected DCs, compared with controls (**Fig. 3b**).

Nanoparticle-complexed siNS1(nano-siNS1) significantly attenuates RSV infection and pulmonary pathology in mice

To determine whether siNS1 exerts an antiviral response *in vivo* in BALB/c mice, we complexed the siNS1 plasmid (10 g per mouse) with a nanochitosan polymer (50 μg), referred to as Nanogene 042 (NG042). We administered the nanoparticles as a nasal drop 2 d before viral inoculation. NS1 expression in the lungs ($n = 6$) of mice was assayed by RT-PCR 18 h after infection. siNS1 substantially knocked down expression of the RSV NS1 gene but not the RSV F gene (**Fig. 4a**). The viral titer in supernatants of homogenized lungs ($n = 8$) was also significantly decreased in the siNS1 treated mice compared to controls ($P < 0.05$; **Fig. 4b**). We challenged these mice ($n = 8$) with methacholine at day 4 following rgRSV infection. RSV-infected mice showed a >400% increase in enhanced pause values compared to baseline and a 300% increase compared to the siNS1 group (**Fig. 4c**). Mice treated with siNS1 showed significantly lower ($P < 0.05$) AHR than that of untreated mice and showed a considerable reduction in pulmonary inflammation, as evidenced by decreases in the goblet cell hyperplasia of the bronchi and in the number of infiltrating inflammatory cells in the interstitial regions compared to controls (**Fig. 4d**). To assess IFN-β expression in the lung tissue of mice treated with siRNA 2d before viral inoculation, we extracted total RNAs from each group of animals, 24 h after infection ($n = 6$ per group) and assayed them by RT-PCR. Knockdown of the RSV NS1 gene significantly increased IFN-β expression in the lung compared to controls ($P < 0.05$; **Fig. 4e,f**). Examination of IFN-α levels in the bronchoalveolar lavage fluid by ELISA showed a twofold increase in IFN concentration in siNS1-treated mice compared to control mice (data not shown).

Table 1 IFN-inducible genes change more than sixfold in RSV-infected A549 cells

GenBank accession number	Gene	Function	Fold change (FC)[a]	Comparison[b] rgRSV	rgRSV + siNS1
NM_007315	STAT1	signal transducer and activator of transcription 1	6	D	I
NM_002198	IRF1	interferon regulatory factor 1	6	D	I
NM_001571	IRF3	interferon regulatory factor 3	6	NC	I
NM_004030	IRF7	interferon regulatory factor 7	6	D	I
NM_006084	IRF9	ISGF3G (p48)	6	D	I
NM_005531	IFI16	interferon gamma-inducible protein 16	6	D	I
NM_005532	IFI27	interferon, alpha-inducible protein 27	6	D	I
NM_006332	IFI30	interferon gamma-inducible protein 30	6	D	I
BF338947	IFITM2	interferon induced transmembrane protein 2	6	D	I
AL121994	1-8U	contains a pseudogene similar to IFITM3 (interferon induced transmembrane protein 3, STSs and GSSs	6	D	I
BE049439	IFI44	interferon-induced, hepatitis C-associated microtubular aggregate protein (44kD)	8	D	I
NM_004509	IFI41	SP110 nuclear body protein (interferon-induced protein 75, 52kD)	6	D	I
NM_003641	PTS	6-pyruvoyltetrahydropterin synthase- interferon induced transmembrane protein 1 (9-27) (IFITM1)	6	D	I
NM_005101	ISG15	interferon alpha-inducible protein (clone IFI-15K)	6	D	I
NM_002201	ISG20	interferon stimulated gene (20kD) (ISG20)	6	D	I
NM_022147	IFRG28	28kD interferon responsive protein	8	D	I
NM_002176	IFNB1	interferon beta 1, fibroblast	8	D	I
NM_002462	MxA	interferon-regulated resistance GTP-binding protein	6	D	I
NM_002463	MxB	interferon-regulated resistance GTP-binding protein	7	D	I
NM_016817	OAS2	2'-5'-oligoadenylate synthetase 2, 69/71kDa	8	D	I
NM_003733	OASL	2'-5'-oligoadenylate synthetase-like	6	D	I
NM_016816	OAS1	2',5'-oligoadenylate synthetase 1, 40/46kDa	6	D	I
NM_006187	OAS3	2'-5'-oligoadenylate synthetase 3, 100kDa	6	D	I
NM_001550	IFRD1	interferon-related developmental regulator 1	6	D	I
NM_001547	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	8	D	I

[a]Value for the fold change in expression calculated by the Microarray Suite 5.0 (MAS 5.0) program. [b]The data were compared to arrays of rgRSV-infected A549 cells either with or without siNS1 treatment. I, increased; NC, not changed; D, decreased.

Potential of Nano-siNS1 for prophylaxis and treatment of RSV infection

To investigate the persistence of siNS1 prophylaxis, we treated mice with the NG042-siNS1 complex at 2, 4 or 7 d before viral inoculation. Analysis of viral titers 5 d after infection showed that the prophylactic effect of siNS1 can last for at least 4 d, although treatment at day −7 still lowers viral titer by 1 $\log_{10}$ compared to the control (**Fig. 5a**). To test whether prophylactic blocking of NS1 activity can induce anti-RSV immunity and provide protection from reinfection, we administered the NG042-siNS1 complex to mice, inoculated them with RSV (5×10^6 plaque-forming units (p.f.u.)/mouse) 2 d later and then reinoculated them with RSV (1×10^7 p.f.u./mouse) after 16 d. Cellular immunity induced by RSV at 5 d after infection was examined in these mice by intracellular cytokine staining of splenocytes for IFN-γ and IL-4. Splenocytes of mice treated with NG042-siNS1 showed an increase in IFN-γ production in both $CD4^+$ and $CD8^+$ T cells and also increases in IL-4 production in $CD4^+$ T cells compared with controls (**Fig. 5b,c**). Examination of virus titer following secondary infection showed that mice treated with NG042-siNS1 showed a significant decrease in the viral titers compared to control mice ($P < 0.05$; **Fig. 5d**). Thus, prophylaxis with siNS1 enhanced cellular immunity and attenuated the secondary RSV infection.

To test the therapeutic potential of NG042-siNS1, we administered the NG042-siNS1 complex to mice at day 0 along with RSV inoculation or at day 2 or 3 after infection. Mice treated the same day as inoculation or at 2 d after RSV infection showed a significantly lower viral titer compared to controls ($P < 0.05$; **Fig. 5e**). Treatment with NG042-siNS1 3 days after inoculation also decreased virus titer, albeit marginally. Further, lung sections of mice treated with NG042-siNS1 2 days after RSV infection showed a substantial decrease in lung inflammation, goblet cell hyperplasia and infiltration of inflammatory cells compared to control mice (**Fig. 5f**).

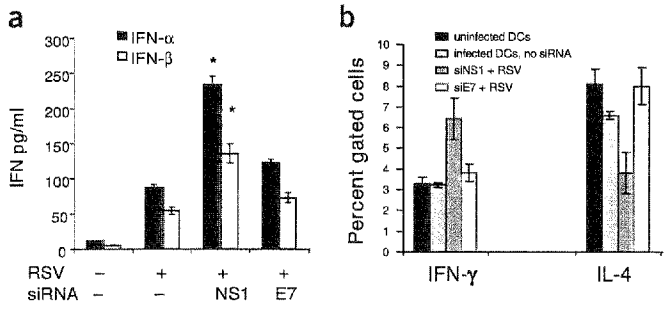

Figure 3 Effect of siNS1 on human DCs and naive CD4+ T cells. (**a**) Expression levels of IFN-α and IFN-β protein in RSV-infected DCs, treated with or without siNS1 were measured by ELISA. $P < 0.05$ for siNS1 versus siE7. (**b**) Flow cytometric analysis of intracellular cytokine production in allogenic naive CD4+ T cells after coculture with RSV-infected DCs, treated with or without siNS1. Results shown are from one representative experiment of three repeats.

DISCUSSION

Although the human RSV NS1 protein has type-1 IFN–antagonistic effects, the mechanism remains unknown. This report underscores the substantial role of NS1 in RSV replication and immunity to RSV infection. These studies show that the NS1 protein downregulates the IFN-signaling system by deactivation of STAT1, IRF1 and IFN-regulated gene expression, which are critical to suppressing IFN action. Furthermore, the results show the potential for nanoparticles encapsulating siNS1 for the prophylaxis and treatment of RSV infections.

Vector-driven *de novo* expression of siRNA to attenuate RSV infection has not yet been reported, although antisense oligonucleotide–mediated attenuation of RSV infection in African green monkeys has been reported[33]. The potential of this approach remains uncertain as the effects of these oligonucleotides were measured at the very early stage of infection (*i.e.*, 30 min after RSV challenge). Mechanistically, both antisense and siRNA work post-transcriptionally to reduce expression of the target gene. The antisense oligonucleotides accumulate in the nucleus and may alter splicing of precursor mRNA[34,35]. In contrast, siRNAs exert their effects in the cytoplasm[36], which is the site of RSV replication. Also, intracellular expression from RNA polymerase III promoters enables the production of stably expressed siRNA in the cell with sustained knockdown of the target, and hence, lower concentrations are needed to achieve levels of knockdown that are comparable to those from antisense reagents.

We demonstrate in this report that DNA-vector driven siNS1 expression is capable of considerably attenuating RSV infection of human epithelial cells, which are the primary targets of RSV replication. We used A549 epithelial cells, as they are similar to cultured primary airway cells in terms of their susceptibility to RSV[37]. The transfection efficiency of the construct as assessed using plasmid pEGFP was 43.21% and 49.62% in A549 and Vero cells, respectively. Despite this, the siNS1 plasmid inhibited rgRSV production by 90–97%, which is consistent with a 2- to 3-$\log10_{10}$ decrease in RSV titers. Furthermore, two different siRNA constructs targeting NS1 showed almost identical results. Although the mechanism of the siNS1-mediated decrease in viral titers was not investigated, it may be attributed to the fact that NS1, located at the 3′ end of the viral genome, acts as a common early-stage promoter for the initiation of both replication and transcription[38]. These results are consistent with reports that suggest that deletion of NS1 strongly attenuates RSV infection *in vivo* [12,14,15] and suggest the potential application of siNS1 for prophylaxis against RSV infection.

We investigated the mechanism of siNS1-induced attenuation of viral replication. To establish that the antiviral effects of siNS1 are the result of the modulation of the IFN pathway, we used Vero cells that lack the type-1 IFN genes and compared them with A549 cells. Whereas A549 cells showed considerable siNS1- or siNS1a-induced decreases in rgRSV-infected cell numbers and virus titers, we saw no effect of siNS1

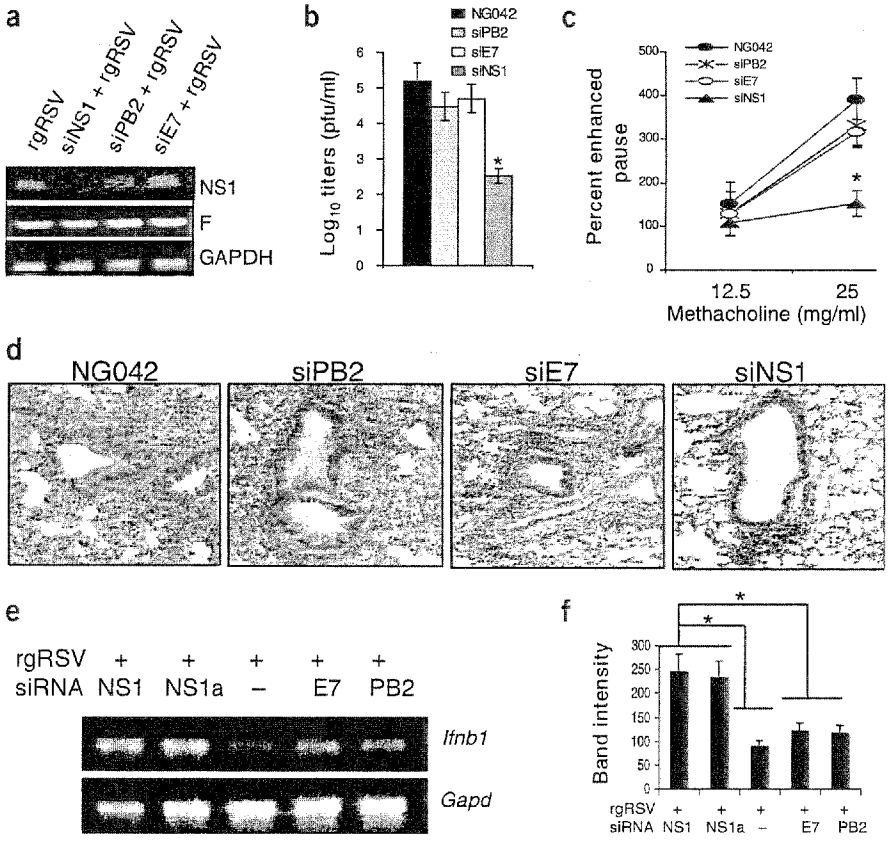

Figure 4 siNS1 exhibits antiviral activity *in vivo*. (**a**) Detection of NS1 gene expression using RT-PCR at 18 h after infection with rgRSV. (**b**) Determination of viral lung titer using plaque assay on A549 cells. $^*P < 0.05$ relative to control. (**c**) Airway responsiveness to inhaled methacholine (MCh) was measured in mice infected with rgRSV following 2 d after prophylaxed with NG042-plasmid complex. The results are expressed as percentage of Penh (enhanced pause) after inhalation of methacholine relative to phosphate-buffered saline. $^*P < 0.05$ compared to control. (**d**) Histology of lung sections of mice treated as in **c** (staining with hematoxylin and eosin). (**e**) Detection of *Ifnb1* gene expression in lung tissue using RT-PCR at 24 h after infection with rgRSV . (**f**) DNA bands were scanned using the Scion image system (US National Institutes of Health) to quantify data in **e**. $^*P < 0.05$ relative to control.

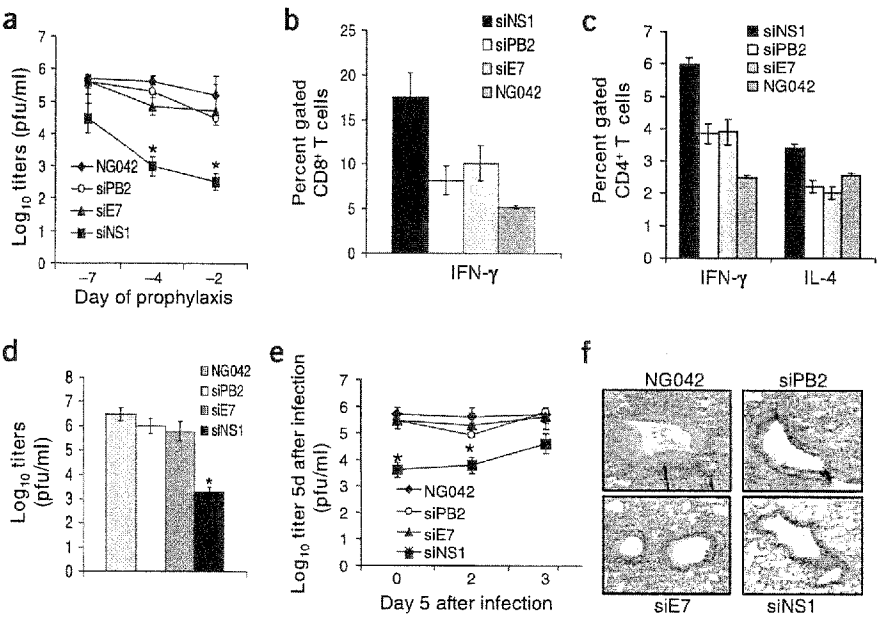

Figure 5 Prophylactic and therapeutic potential of NG042-siNS1. (**a**) Measurement of viral lung titer in the mice given prophylactic treatment at 2, 4 or 7 d before RSV infection using plaque assay on A549 cells. $^*P < 0.05$ relative to control. (**b**,**c**) Intracellular cytokine production in spleen T cells in the mice at 5 d after secondary infection, which were administered prophylactic treatment at day −2, inoculated with rgRSV at day 1 and day 16. (**d**) Measurement of viral lung titer from rechallenged mice (1×10^7 p.f.u./mouse) at day 5 after secondary infection. $^*P < 0.05$ compared to control. Results of one experiment of two representative experiments are shown. (**e**) Analysis of lung RSV titers at 5 d after infection by plaque assay on A549 cells of mice treated with siRNA after different days of rgRSV-inoculation as indicated. $^*P < 0.05$ relative to control. (**f**) Histology (staining with hematoxylin and eosin) of lung sections of mice treated with NG042-siNS1s, control siRNAs, or NG042 alone, at day 2 after infection.

or siNS1a in Vero cells. Also, in parallel studies, Vero cells cotransfected with pEGFP and siEGFP, not siNS1, showed substantial knockdown (91.68%) of EGFP gene expression (data not shown). These results show a definitive role of siNS1 and siNS1a in the attenuation of RSV replication and implicate the type-1 IFN pathway in this process.

IFNs drive a cascade of intracellular signaling, resulting in the expression of a large number of interferon-stimulated genes (ISGs) that exert the pleiotropic effects of IFN, including interference with viral replication and modulation of the host immune response[39]. The level of expression of IFN-inducible genes in infected A549 cells treated with siNS1 was considerably altered, as shown by the microarray data. IRF3 and MxA expression were upregulated after NS1 inhibition, in agreement with a previous report on bovine RSV[26], although STAT2 levels were not changed. In addition, expression of STAT1, IRF1, and ISGF-3γ, were substantially upregulated in our studies compared to control. IRF1 may have an important role in human RSV infection because it functions as a transcriptional activator[40] and binds to the positive regulatory domain 1 of the IFN-β promoter[41] and to the IFN-stimulated response element of IFN-stimulated genes[42]. ISGF-3γ encodes a protein-interaction function that allows recruitment of STAT1 and STAT2, their translocation from the cytoplasm to the nucleus and initiation of transcription of IFN-stimulated genes[39]. Furthermore, results show that both the IRF1 and phosphorylated STAT1 proteins translocate to the nucleus of infected A549 cells through knockdown of the NS1 protein, which suggests that NS1 targets activation of STAT1 and IRF1.

An important finding of this study is that siNS1 and siNS1a induced substantially higher amounts (a threefold increase) of IFN-β compared to controls, including siE7 or siPB2 (expressed from the same plasmid vector backbone as siNS1) and the unrelated siRNA, indicating that NS1 is involved in antagonizing type-1 IFN. These results are in agreement with the increases in IFN production observed with NS1/NS2-deleted human RSV infection[25–28]. It is noteworthy, however, that compared to RSV-infected cells, cells transfected with either the vector plasmid or with siRNA targeting different viral antigens or an unrelated siRNA showed a slight increase of IFN-β production following RSV infection. This may be attributable to plasmid-driven siRNA-induced IFN-stimulated genes, including PKR and OAS[43,44], to CpG motifs (*amp*^{r} gene) present in the vector plasmid that activate innate immunity by binding to TLR9 (ref. 45), or to the U6 promoter-vector, which induces a higher frequency of interferon-stimulated genes compared to lentiviral H1 vectors[46]. The vector or control siRNA–induced IFN production also upregulates certain IFN-inducible genes, particularly those encoding STAT1 and IRF1 and IRF3, which might account for the finding that siE7 or siPB2 reduced rgRSV production by about 1 $\log_{10}$ *in vitro*. But siNS1 induces a considerably higher level of expression of these ISGs, including MxA and ISGF-3γ, and, in addition, promotes phosphorylation of STAT1.

Whereas epithelial cells are the major target cells in which the virus replicates, monocytes and DCs have a role in generating anti-RSV immunity. Monocytes have a role in the pathophysiology of RSV bronchiolitis[47], and they represent a pool of circulating precursors capable of differentiating into DCs that are able to present pathogen-derived peptides to naive T cells. NS1 seems to decrease type-1 IFN production in DCs, presumably affecting their state of activation and antigen presentation. The results of these studies show that RSV infection decreases the capacity of DCs to induce IFN-γ in naive T cells[48], which might cause the delayed RSV-specific immune response and permit multiple RSV reinfections. Thus, infected DCs treated with siNS1 produce much more type-1 IFN and also drive naive CD4$^+$ T cells toward T_H1-type lymphocytes that generate more IFN-γ and less IL-4.

The effects of siRNA have been amply shown in cultured cells. But only a few studies have addressed the potential of siRNA-based therapeutics *in vivo* using model animal systems. A notable result of this report is that a new generation of oligomeric nanometer-size chitosan particles, NG042, can be used for *de novo* expression of siNS1 in the lung tissues that results in protection from RSV infection. NG042 shows higher transduction efficiency and induces less inflammation compared to classical high molecular weight chitosan (data not shown). The results of studies on the prophylactic potential of NG042-siNS1 indicate that siNS1 induces substantial protection from rgRSV infection, infection-induced inflammation and airway reactivity, and the protective effect lasted for at least 4 d. Furthermore, even a single-dose prophylaxis with NG042-siNS1 considerably inhibits reinfection in mice that are administered a higher dose of RSV 16 d after primary infection. The precise mechanism of enhanced protection is unknown, but it is probable that knockdown of the NS1 gene augments anti-RSV host immunity through enhanced IFN production and thereby prevents

mice from RSV reinfection. In addition, NG042-siNS1 also attenuates established RSV infection. Thus, the antiviral treatment decreased viral titer in the lung, improved pulmonary function and attenuated pulmonary inflammation in rgRSV-infected mice.

In conclusion, our data show that NS1 promotes virus infection of human epithelial and dendritic cells by inhibiting the type-1 IFN pathway. Treatment with NG042-siNS1 either before or after RSV infection substantially attenuates RSV infection and infection-induced pulmonary pathology in mice. Thus, siNS1 nanoparticles may prove to be a potent, new prophylactic and/or therapeutic agent against RSV infection in humans.

METHODS

Virus and cell lines. A549, Vero cell line and RSV strain A2 were obtained from the American Type Culture Collection. Recombinant rgRSV which encodes green fluorescent protein was supplied by M. E. Peeples[31].

Plasmid constructs. The nucleotide sequence for each siRNA is as follows: siNS1: 5′-GGCAGCAATTCATTGAGTATGCTTCTCGAAATAAGCATACTCA ATGAATTGCTGCCTTTTTG-3′; siNS1a: 5′-GTGTGCCCTGATAACAATAT TCAAGAGATATTGTTATCAGGGCACACTT-TTTTG-3′; siE7: 5′-GAAAAC GATGAAATAGATGTTCAAGAGACATCTATTTCATCGTTTTCT-TTTTT-3′; siPB2: 5′-GGCTATATTCAATATGGAAAGAACTCGAGTTTTGTTCTTTCCA TATT-GAATATAGCCTTTTTG-3′; and siUR: 5′-GGTCACGATCAGAATACT TCGCTCGAGCGAAG-TATTCTGATCGTGACCCTTTTTTG-3′. Each pair of oligonucleotide sequences was inserted into pSMWZ-1 plasmid at appropriate sites respectively, to generate the corresponding siRNA for RSV NS1, HPV_{18} E7, type A Influenza virus PB2 and pUR.

DNA transfection and virus infection. Cells were transfected with siNS1 or controls (siE7, siPB2 or siUR) using Lipofectamine 2000 reagent (Invitrogen). We infected cells 24 h later with rgRSV or RSV at appropriate multiplicity of infection. We used the pEGFP plasmid (Stratagene) for measurement of transfection efficiency.

Flow cytometry. To quantify rgRSV-infected cells, cells were harvested and scored for GFP-positive cells by flow cytometry with appropriate gating and proper controls at the Moffitt Flow Cytometry Core. Additional flow cytometry analyses were conducted utilizing fluorescent-labeled antibodies.

Isolation of DCs from human peripheral blood and measurement of IFNs in supernatants of infected DCs. Monocytes purified from PBMCs using monocyte isolation Kit II (Miltenyi Biotec.) were seeded into 6-well culture plates supplemented with 200 ng/ml IL-4 and 50 ng/ml GM-CSF (BD Pharmingen) and cultured for 6–7 d for plasmid transfection and infection with RSV. We assayed expression of IFNs in the supernatants by IFN-α Multi-Species ELISA Kit and IFN-β ELISA kit (PBL Biomedical Laboratories).

Analysis of intracellular cytokine production in T cells. Human naive $CD4^+$ T cells (1×10^6 cells/well) purified using $CD4^+$ T cell isolation kit (Miltenyi Biotec.) from umbilical cord blood were cocultured with irradiated monocyte-derived DCs (30 Gy) (1×10^5 cells/well) in 24-well plates for 6 d with additional culture for 8 d in the presence of recombinant human IL-2 (10 ng/ml). We cultured mouse spleen T cells purified using mouse T-cell enrichment column kit (R & D Systems) in 6-well plates for 4 d. Finally, cells were stimulated with phorbol-12-myristate-13-acetate (50 ng/ml) and ionomycin (500 ng/ml) (Sigma) for 6 h in the presence of GolgiStop (PharMingen) and then fixed and stained them using CD8 or CD4 monoclonal antibody (BD Biosciences) for flow cytometry analysis.

Immunofluorescence. A549 cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100 and blocked with 3% donkey serum in phosphate-buffered saline containing 1% glycerin for 60 min. We next incubated cells with IRF1 antibody (Santa Cruz Biotec.) or phosphorylated STAT1 (Ser727; Upstate), respectively, and then with Zenon Alexa Fluor 488 (Molecular Probes). The slides were visualized by immunofluorescence microscopy.

Plaque assay. We added tenfold serial dilutions of the supernatants to a monolayer of A549 cells and replaced the medium in each well of six-well culture plates by an agarose-containing overlay (2× DMEM, 10% fetal bovine serum, 1% low melting point agarose (Gibco BRL)). The plates were incubated at 37 °C for 5 d. Afterward, we added 1% neutral red (Sigma) and counted the plaques 48 h later.

Microarray assays. Total RNAs were extracted by Qiagen RNeasy Kit. We used 10 μg of total RNAs to prepare cDNA. Gene expression was analyzed with GeneChip Human Genome U95Av2 probe array (Affymetrix) according to the manufacturer's protocol (Expression Analysis Technical Manual). We performed data analysis with Microarray Suite 5.0 (MAS 5.0).

Protein expression analysis by western blotting. Transfected A549 cells were infected with rgRSV (MOI = 1). We performed electrophoresis on the whole cell lysates using 12% polyacrylamide gels and transferred the proteins to PVDF membranes (BIO-RAD). The blot was incubated separately with RSV polyclonal antibody (AB1128, Chemicon Int.), STAT1, phoshphorylated STAT1 (Tyr701), STAT2, IRF1, IRF3, IRF7, ISGF-3γ and IFN-β (Santa Cruz), phosphorylated STAT1 (Ser727, Upstate) or MxA antibody (O. Haller). Immunoblot signals were developed by Super Signal Ultra chemiluminescent reagent (Pierce).

Studies in mice. Animal studies were approved by the University of South Florida and Veterans' Affairs Hospital Institutional Animal Care and Utilization Committee. All animal studies were blinded to remove investigator bias. We administered plasmid (10 μg per mouse) with NG042 (50 μg per mouse) (TransGenex Nanobiotech Inc) intranasally to 6-week-old female BALB/c mice (Charles River, $n = 8$ per group) before or after inoculation with rgRSV (5×10^6 p.f.u./mouse). The pulmonary function was evaluated at day 4 after infection as described previously[21]. Finally, all mice were killed the next day. The RSV titer was determined by plaque assay from the lung homogenate ($n = 8$), and histological sections from lungs ($n = 8$) were stained with hematoxylin and eosin. We performed RT-PCR analysis in the lung tissue using the following primers. IFN-β: forward, 5′-ATAAGCAGCTC-CAGCTCCAA-3′, reverse, 5′-CTGTCTGCTGGTGGAGTTCA-3′; RSV-NS1: forward, 5′-ATGGGGTGCAATTCATTGAG-3′, reverse, 5′-CAGGGCACAC TTCACTGCT-3′; RSV-F: forward, 5′-TGCAGTGCAGTTAGCAAAGG-3′, reverse, 5′-TCTGGCTCGATTGTTTGTTG-3′; and GAPDH: forward, 5′-CCCTTCATTGACCTCAACT-3′, reverse, 5′-GACGCCAGTG-GACTCCA-3′. PCR products were visualized by gel electrophoresis and quantified by densitometry.

Statistical analysis. Pairs of groups were compared by Student's t-test. Differences between groups were considered significant at $P < 0.05$. Data for all measurements are expressed as means ± s.d.

ACKNOWLEDGEMENTS

We thank M.E. Peeples (Columbus Children's Research Institute, Ohio) for his rgRSV stock, O. Haller (Freiburg University, Germany) for his gift of MxA antibody, Saneron-Cell Therapeutics (Tampa, Florida) for the cord blood cells and Moffitt Flow Cytometry and Microarray Core for their assistance. SSM is supported by the grants from the Veterans' Affairs Merit Review Award and US National Institutes of Health #5RO1HL71101-01A2. The support from the Joy McCann Culverhouse Endowment to the Division of Allergy and Immunology, Department of Internal Medicine, College of Medicine, and the Veterans' Affairs Hospital is also gratefully acknowledged.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Medicine* website for details).

Received 25 May; accepted 2 December 2004
Published online at http://www.nature.com/naturemedicine/

1. Sigurs, N., Bjarnason, R., Sigurbergsson, F. & Kjellman, B. Respiratory syncytial virus bronchiolitis in infancy is an important risk factor for asthma and allergy at age 7. *Am. J. Respir. Crit. Care Med.* **161**, 1501–1507 (2000).
2. Sigurs, N., Bjarnason, R., Sigurbergsson, F., Kjellman, B. & Bjorksten, B. Asthma and immunoglobulin E antibodies after respiratory syncytial virus bronchiolitis: a prospective cohort study with matched controls. *Pediatrics* **95**, 500–505 (1995).
3. Shay, D. K. *et al.* Bronchiolitis-associated hospitalizations among US children, 1980-1996. *JAMA* **282**, 1440–1446 (1999).
4. Hall, C. B., Long, C. E. & Schnabel, K. C. Respiratory syncytial virus infections in previ-

ously healthy working adults. *Clin. Infect. Dis.* **33**, 792–796 (2001).
5. Thompson, W. W. *et al.* Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA* **289**, 179–186 (2003).
6. Sly, P. D. & Hibbert, M. E. Childhood asthma following hospitalization with acute viral bronchiolitis in infancy. *Pediatr. Pulmonol.* **7**, 153–158 (1989).
7. Brandenburg, A. H., Neijens, H. J. & Osterhaus, A. D. Pathogenesis of RSV lower respiratory tract infection: implications for vaccine development. *Vaccine* **19**, 2769–2782 (2001).
8. Coffin, S. E. & Offit, P. A. New vaccines against mucosal pathogens: rotavirus and respiratory syncytial virus. *Adv. Pediatr. Infect. Dis.* **13**, 333–348 (1997).
9. Handforth, J., Friedland, J.S. & Sharland, M. Basic epidemiology and immunopathology of RSV in children. *Paediatr. Respir. Rev.* **1**, 210–214 (2000).
10. Welliver, R.C. Review of epidemiology and clinical risk factors for severe respiratory syncytial virus (RSV) infection. *J. Pediatr.* **143**, S112–S117 (2003).
11. Collins, P.L., Chanock, R.M. & Murphy, B.R. in *Fields Virology* Vol. 1, 4th edn (eds. Knipe, D.M., Howley, P.M. & Griffin, D.E.), 1443–1485 (Lippincott-Raven, Philadelphia, 2001).
12. Jin, H. *et al.* Recombinant respiratory syncytial viruses with deletions in the NS1, NS2, SH, and M2-2 genes are attenuated in vitro and in vivo. *Virology* **273**, 210–208 (2000).
13. Teng, M. N, & Collins, P. L. Altered growth characteristics of recombinant respiratory syncytial viruses which do not produce NS2 protein. *J. Virol.* **73**, 466–473 (1999).
14. Teng, M. N. *et al.* Recombinant respiratory syncytial virus that does not express the NS1 or M2-2 protein is highly attenuated and immunogenic in chimpanzees. *J. Virol.* **74**, 9317–9321 (2000).
15. Murphy, B.R, & Collins, P.L. Live-attenuated virus vaccines for respiratory syncytial and parainfluenza viruses: applications of reverse genetics. *J. Clin. Invest.* 110, 21-27 (2002).
16. Hall, C.B., Walsh, E.E., Long, C.E. & Schnabel, K.C. Immunity to and frequency of reinfection with respiratory syncytial virus. *J. Infect. Dis.* **163**, 693–698 (1991).
17. Roman, M. *et al.* Respiratory syncytial virus infection in infants is associated with predominant T_H2-like response. *Am. J. Respir. Crit. Care Med.* **156**, 190–195 (1997).
18. Matsuse, H. *et al.* Recurrent respiratory syncytial virus infections in allergen-sensitized mice lead to persistent airway inflammation and hyperresponsiveness. *J. Immunol.* **164**, 6583–6592 (2000).
19. Behera, A. K., Kumar, M., Lockey, R. F. & Mohapatra, S. S. Adenovirus-mediated interferon gamma gene therapy for allergic asthma: involvement of interleukin 12 and STAT4 signaling. *Hum. Gene Ther.* **13**, 1697–1709 (2002).
20. Kumar, M., Behera, A. K., Matsuse, H., Lockey, R. F. & Mohapatra, S. S. Intranasal IFN-γ gene transfer protects BALB/c mice against respiratory syncytial virus infection. *Vaccine* **18**, 558–567 (1999).
21. Kumar, M. *et al.* Intranasal gene transfer by chitosan-DNA nanospheres protects BALB/C mice against acute respiratory syncytial virus infection. *Hum. Gene Ther.* **13**, 1415–1425 (2002).
22. Kumar, M. *et al.* Chitosan IFN-g-pDNA nanoparticle (CIN) therapy for allergic asthma. *Genetic Vaccines and Ther.* **1**, 3–12 (2003).
23. Mohapatra, SS. Mucosal gene expression vaccine: a novel a vaccine strategy for respiratory syncytial virus. *Pediatr. Infect. Dis J.* **22**, S100–S103 (2003).
24. Hellermann, G., Mohapatra, SS. Genetic Therapy: on the brink of a newfuture. *Genetic Vaccines and Ther.* **1**, 1–3 (2003).
25. Bossert, B. & Conzelmann, K.K. Respiratory syncytial virus (RSV) nonstructural (NS) proteins as host range determinants: a chimeric bovine RSV with NS genes from human RSV is attenuated in interferon-competent bovine cells. *J. Virol.* **76**, 4287–4293 (2002).
26. Bossert. B., Marozin, S. & Conzelmann, K.K. Nonstructural proteins NS1 and NS2 of bovine respiratory syncytial virus block activation of interferon regulatory factor 3. *J. Virol.* **77**, 8661–8668 (2003).
27. Schlender, J., Bossert, B., Buchholz, U, & Conzelmann, K.K. Bovine respiratory syncytial virus nonstructural proteins NS1 and NS2 cooperatively antagonize α/β interferon-induced antiviral response. *J. Virol.* **74**, 8234–8242 (2000).
28. Spann, K.M., Tran, K.C., Chi, B., Rabin, R.L. & Collins, P.L. Suppression of the induction of α, β, and γ interferons by the NS1 and NS2 proteins of human respiratory syncytial virus in human epithelial cells and macrophages. *J. Virol.* **78**, 4363–4369 (2004).
29. Fire, A. RNA-triggered gene silencing. *Trends Genet.* **15**, 358–363 (1999).
30. Zhang, W., Singam, R., Hellermann, G., Kong, X., San Juan, H., Lockey, R.F., Wu, S.J., Porter, K., Mohapatra, S.S. Attenuation of dengue virus infection by adeno- associated virus-mediated siRNA delivery. *Genetic Vaccines Ther.* **2**, 8–12 (2004).
31. Haliak, L.K., Collins, P.L., Knudson, W. & Peeples, M.E. Iduronic acid-containing glycosaminoglycans on target cells are required for efficient respiratory syncytial virus infection. *Virology* **271**, 264–275 (2000).
32. Mosca, J.D. & Pitha, P.M. Transcriptional and posttranscriptional regulation of exogenous human β interferon gene in simian cells defective in interferon synthesis. *Mol. Cell. Biol.* **6**, 2279–2283 (1986).
33. Leaman, D.W. *et al.* Targeted therapy of respiratory syncytial virus in African green monkeys by intranasally administered 2-5A antisense. *Virology* **292**, 70–77 (2002).
34. Fisher, T.L., Terhorst, T., Cao, X. & Wagner, R.W. Intracellular disposition and metabolism of fluorescently-labeled unmodified and modified oligonucleotides microinjected into mammalian cells. *Nucleic Acids Res.* **21**, 3857–3865 (1993)
35. Kole, R. & Sazani, P. Antisense effects in the cell nucleus: modification of splicing. *Curr. Opin. Mol. Ther.* **3**, 229–234 (2001).
36. Billy, E., Brondani, V., Zhang, H., Muller, U. & Filipowicz, W. Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. *Proc. Natl. Acad. Sci. USA* **98**, 14428–14433 (2001).
37. Arnold, R., Humbert, B., Werchau, H., Gallati, H. & Konig, W. Interleukin-8, interleukin-6, and soluble tumor necrosis factor receptor type-I release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus. *Immunology* **82**,126–133 (1994).
38. Atreya, P.L., Peeples, M.E. & Collins, P.L. The NS1 protein of human respiratory syncytial virus is a potent inhibitor of minigenome transcription and RNA replication. *J. Virol.* **72**, 1452–1461 (1998).
39. Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. & Schreiber, R. D. How cells respond to interferons. *Annu. Rev. Biochem.* **67**, 227–264 (1998).
40. Barnes, B., Lubyova, B. & Pitha, P.M. On the role of IRF in host defense. *J. Interferon Cytokine Res.* **22**, 59–71 (2002).
41. Harada, H. *et al.* Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. *Cell* **58**, 729–739 (1989).
42. Pine, R., Decker, T., Kessler, D.S., Levy, D.E. & Darnell, JE Jr. Purification and cloning of interferon-stimulated gene factor 2 (ISGF2): ISGF2 (IRF-1) can bind to the promoters of both beta interferon- and interferon-stimulated genes but is not a primary transcriptional activator of either. *Mol. Cell. Biol.* **10**, 2448–2457 (1990).
43. Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H. & Williams, B.R. Activation of the interferon system by short-interfering RNAs. *Nat. Cell Biol.* **5**, 834–839 (2003).
44. Bridge, A. J., Pebernard, S., Ducraux, A., Nicoulaz, A. L., & Iggo, R. Induction of an interferon response by RNAi vectors in mammalian cells. *Nat. Genet.* **34**, 263–264 (2003).
45. Sato, Y. *et al.* Immunostimulatory DNA sequences necessary for effective intradermal gene immunization. *Science* **273**, 352–354 (1996).
46. Pebernard, S., & Iggo, R.D. Determinants of interferon-stimulated gene induction by RNAi vectors. *Differentiation* **72**, 103–111 (2004).
47. Bont, L., Kavelaars, A., Heijnen, C.J., van Vught, A.J. & Kimpen, J.L. Monocyte interleukin-12 production is inversely related to duration of respiratory failure in respiratory syncytial virus bronchiolitis. *J. Infect. Dis.* **181**, 1772–1775 (2000).
48. Bartz, H. *et al.* Respiratory syncytial virus decreases the capacity of myeloid dendritic cells to induce interferon-gamma in naive T cells. *Immunology* **109**, 49–57 (2003).